Influence of Phosphate and Other Factors on the Rennin Gel Obtained with Whole Casein and with κ-Casein in the Presence of Calcium Salts

C. A. ZITTLE
Eastern Utilization Research and Development Division
Agricultural Research Service, USDA
Philadelphia, Pennsylvania 19118

Abstract

Firm gels are obtained by the action of rennin on solutions of whole casein (0.5%) containing calcium chloride (0.010 M) and sodium phosphate (0.0030 M); without the phosphate, flocculent precipitates are obtained. κ-Casein acted on by rennin gives gels irrespective of the type of salts present. The phosphate effects gel formation with whole casein at molar ratios of phosphate to calcium chloride as little as 1:24. Glycerophosphate, substituted phosphate, is not effective in gel formation nor is the sulfate ion. Polyphosphate-hexameta (7) and imidazole prevent or lessen gel formation, presumably because micelle formation is decreased, judged by visual changes in turbidity. Reduction of gel formation by sodium chloride (0.05 M) is also indicated by reduction in micelle formation. It could only be concluded that the phosphate-calcium bond, presumably ionic, is no weaker than the calcium-casein bond. Exposure of casein solutions to HCl vapors gives gels characterized by the low pH (1.0) of formation.

Addition of sodium chloride to whole casein solutions reduced viscosity; further addition of calcium chloride reduced viscosity more. The latter is a specific effect accompanying micelle formation. κ-Casein alone does not show this effect. Addition of phosphate to the whole casein calcium–containing system does not give the expected increase in viscosity but a slight decrease instead, supporting the theory that the surface of the casein micelle does not favor calcium-phosphate cross bonds but that the action of rennin leads to the appearance of a reactive surface.

Introduction

The need for phosphate to obtain a firm clot when rennin acts on casein in the presence of calcium salts was first observed by Hammarsten (1) and also more recently by others (9). Our studies were undertaken to amplify these observations and also to determine whether the influence of phosphate on casein solutions is evident before clot formation. Comparisons were made of clots with whole casein and with κ-casein.

Materials and Methods

κ-Casein was prepared from pooled milk by the urea–sulfuric acid method and has been described (8).

a_s-Casein was prepared from pooled milk by a modified urea procedure (10) followed by a final purification with ethanol (11).

Whole casein was acid precipitated and was principally that used in previous experiments (6).

Viscosity measurements were with an Ostwald type viscometer in a water bath held at 29.5 C with flow times with water of about 60 seconds. Small portions (0.1 or 0.2 ml) of salt solutions (2.0 M NaCl, 1.0 or 4.0 M $CaCl_2$; 0.4 M sodium phosphate, pH 6.8) were added to 12.0 ml of the casein solutions. pH was read after the salts were added. Ten milliliters of solution were placed in the viscometer.

Clotting experiments. A stock solution of 1.0% casein in water, adjusted to pH 7.5 with NaOH, was usually used. Stock solutions of 0.1 M $CaCl_2$ and of 0.1 M Na_2HPO_4 pH 6.8 were used. The final volume for the test was usually 5.0 ml. Final adjustments of pH were made with 0.02 M HCl or NaOH so that in comparative tests the pH values were identical at pH 6.2 to 6.4. Tests were done at 25 C or 30 C. Commercial rennin was added at a weight ratio of 1:250. Visual observations of clotting behavior were made for several hours and finally at 18 hours.

Results

Rennin clotting of caseins. The influence of phosphate on the rennin clotting of solutions of whole casein containing calcium chloride was determined visually. Results of a comparative

experiment are shown in Figure 1. In this particular experiment the casein was 0.43%, the pH 6.4, the $CaCl_2$ 0.010 M, and the sodium phosphate 0.0035 M. Temperature was 25 C. The results illustrated were obtained 2 hours after the rennin was added. The gel in the right tube was still firm after 18 hours, whereas the flocculent precipitate in the left tube (no added phosphate) occupied only a small volume on the bottom and sides of the tube. Casein solutions of 0.26, 0.68, and 1.36% in similar experiments yielded comparable results; however, the gel with 0.26% casein broke when the tube was inverted. The phosphate concentration could be varied widely and yet be effective in gel formation. The phosphate was varied from 0.00042 M to 0.0102 M (6 levels) in the standard system described. Firm gels were obtained in each instance. When the tubes were inverted, the gel with the lowest phosphate was the first to break. However, glycerophosphate could not substitute for phosphate. The closely related arsenate anion did not give a gel but the precipitate with rennin remained in suspension. The divalent sulfate anion did not yield a gel. Polyphosphate-hexameta (7) could not substitute for phosphate in the standard test; the polyphosphate (0.02%), however, bound or displaced the Ca^{++} and the solution was almost water clear, thus producing a system that showed no visible effects of rennin action. Imidazole (0.02 M) in a system containing both $CaCl_2$ and phosphate reduced the gel strength. With imidazole a reduction in micelle formation was evident by visual observation of relative turbidity.

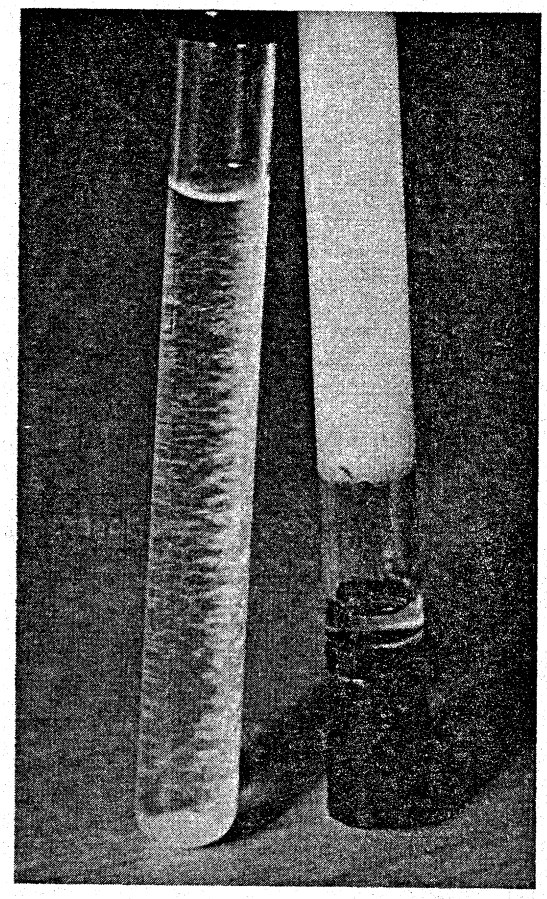

FIG. 1. Influence of phosphate on the rennin clotting of whole casein. Concentration of reagents: Casein, 0.43%; $CaCl_2$, 0.010 M; Na_2HPO_4 (in right tube only), 0.0035 M; rennin, 0.1 mg per 5.8 ml.

Since the effect of phosphate on rennin gelling of casein might involve ionic bonds, the influence of NaCl on the reaction was studied. Additions of NaCl to the standard test up to 0.035 M had no influence on gel formation or on micelle formation, judged by no change in opacity of the calcium ion–containing solutions. With 0.052 M NaCl the opacity with calcium ion was slightly reduced and gel strength weakened. With 0.086 M NaCl, opacity was greatly reduced; with 0.173 M NaCl the solution remained clear; no gels were obtained.

Both α_s-casein and β-casein solutions (0.5%) containing κ-casein (1:5) reacted as did whole casein in the standard test. The solutions containing phosphate gelled with rennin, whereas the solutions without phosphate flocculated and adhered to the sides or settled in the tubes.

κ-Casein alone (0.43%) when examined in the standard test showed little difference between solutions containing $CaCl_2$ alone and those containing the chloride plus phosphate. Both solutions gelled with rennin. The gels were somewhat softer than those with whole casein but the gel containing phosphate could not be distinguished from that without. Similar gels were obtained in water alone (pH 6.0) or with either phosphate (0.010 M) or NaCl (0.035 M), thus appearing characteristic of para-κ-casein whatever the salts (with these salts alone no visual changes occur when rennin acts on whole casein).

Because of the difference in the clots with κ-casein and whole casein, α_s-casein was added to κ-casein solutions containing $CaCl_2$ to determine at what point the transition between gels and flocculent precipitates occurred when these mixtures were acted on by rennin. In these experiments the total protein was 0.6%, but the ratio of κ to α_s was varied. With ratios of 1:8 and 1:2 no gel was obtained with $CaCl_2$ alone, but firm gels were obtained when phosphate was

present also. When the κ:α_s ratio was 3:1, soft gels were obtained with $CaCl_2$ alone, similar to the κ-casein gels, but the gels with phosphate were much firmer.

Exposure of casein solutions to HCl vapors has been reported to give gels. Presumably, the quiet introduction of the acid vapor side-steps coagulation. Gel formation was readily confirmed with a solution (1.4%) of whole casein placed over concentrated HCl in a closed jar. This was not an isoelectric gel since the final pH was 1.0. Visually the reaction showed a sequence of steps: streamers of opalescence appeared, then the liquid became white; next, clearing was apparent and, finally, the liquid became white again and the gel was apparent in 30 to 45 minutes. These changes probably reflect the drop in pH with uptake of HCl vapors. The coagulum by stirring the gel was soluble at pH 7.0 but it was not soluble at pH 3.0, at which the untreated casein was soluble. Additions of $CaCl_2$ (0.01 M) or sodium phosphate (0.0033 M) had no influence on this type of gel. α_s, β-, and κ-caseins were given the same treatment. β-Casein gelled most rapidly, whereas α_s- and κ-caseins reacted much slower. α_s-Casein gave the firmest gel, whereas the β- and κ-casein gels were flocculent.

Previous experiments (14) had indicated that the volume of the sedimented clot of para-κ-casein from heated κ-casein (calcium chloride present) was greater than that from unheated κ-casein. This could not be confirmed with the purer κ-casein now available. Also, κ-casein heated with NaCl, which presumably leads to aggregation of the heated κ-casein (8), showed no difference from the unheated in clot volume. The difference between clotting times of heated and unheated κ-casein was less than 20%, whereas previously (8) the unheated κ-casein was more than twofold slower.

Viscosity of casein with various salts. The influence of salts on the viscosity of whole casein and κ-casein is shown in Figures 2 and 3. Sodium chloride (0.033 M; doubling this concentration gave little or no further effect) decreased viscosity of both caseins by reducing charge repulsion and elongation of the casein molecules. Calcium chloride has no further effect on the viscosity of κ-casein, but it does reduce further the viscosity of whole casein. Subsequent addition of phosphate had little or no influence on the viscosity of κ-casein but phosphate consistently lowered further, to a small degree, the viscosity of whole casein. A small difference in the same direction was also obtained with 0.7% whole casein containing 0.0095 M $CaCl_2$ compared with the same solution containing 0.0032 M sodium phosphate in addition.

The time course of the viscosity changes when rennin acts on whole casein was studied at pH 6.4, 1.0% concentration, with $CaCl_2$ (0.010 M) alone and with $CaCl_2$ plus phosphate (0.0030 M). In both cases there was an initial drop in viscosity followed by a rise and clotting, similar to the curves with casein in milk (3). In no case were there differences in the general shape of the curve that could be attributed to the phosphate. Clotting was faster in the presence of phosphate. Similar viscosity curves were obtained with κ-casein (0.5%) with $CaCl_2$ and with $CaCl_2$ plus phosphate.

Discussion

Firm gels were readily obtained by the action of rennin on 0.43% whole casein at pH 6.4 with 0.010 M $CaCl_2$ and 0.0035 M phosphate. Without phosphate flocculent precipitates were obtained instead of gels. The contribution of $CaCl_2$ to this system is by way of micelle formation. Without $CaCl_2$ the solutions are water clear and the action of rennin produces no visible changes. Even with 0.0033 M $CaCl_2$ the solutions are only faintly opalescent and there is little evidence of rennin action; with 0.0067 M $CaCl_2$ the solutions are strongly opalescent but no gel is obtained. With 0.010 M $CaCl_2$

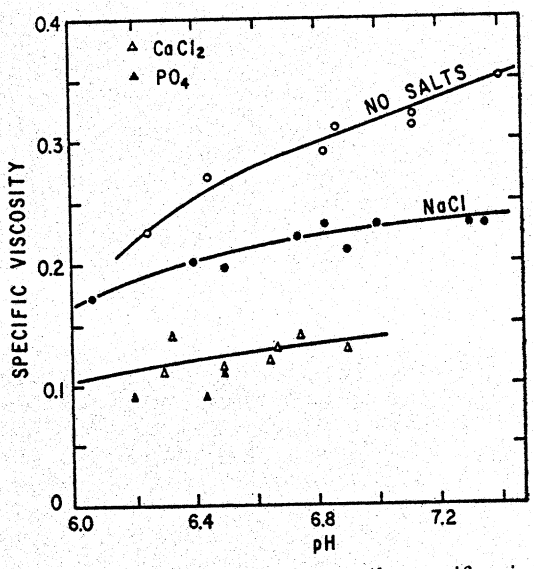

FIG. 2. Influence of salts on the specific viscosity of 1.2% solutions of whole casein at various pH values at 29.5 C. ○ Viscosity with no added salts; ● viscosity with 0.033 M NaCl present; △ viscosity with 0.016 or 0.033 M $CaCl_2$ in addition to NaCl; ▲ viscosity with 0.0064 M Na_2HPO_4 in addition to 0.016 M $CaCl_2$ and NaCl.

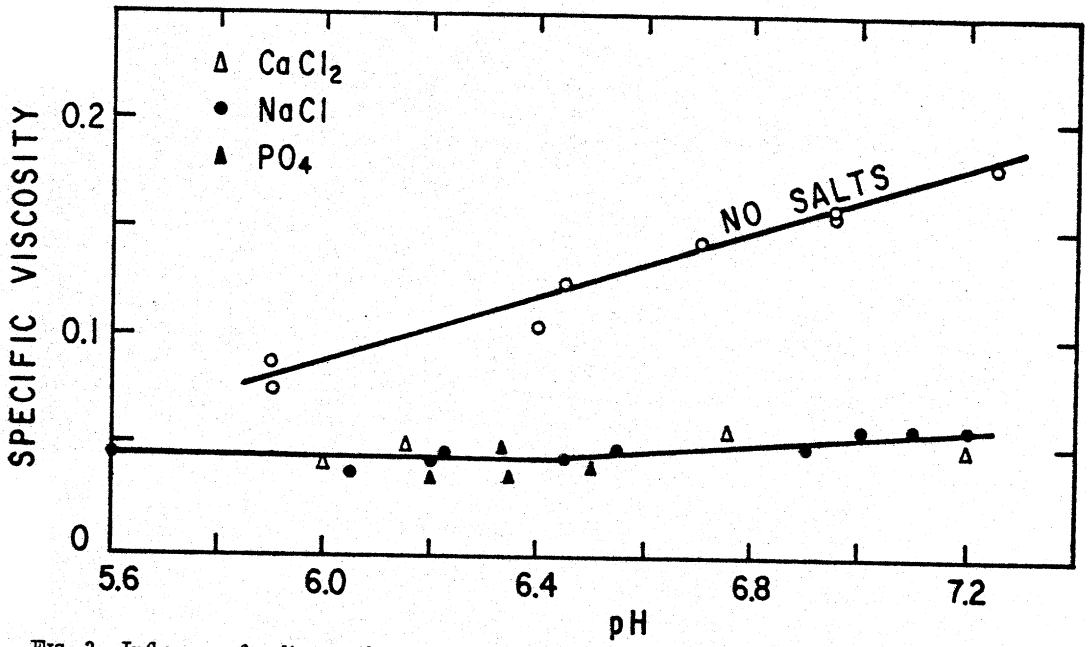

FIG. 3. Influence of salts on the specific viscosity of 0.45% solutions of κ-casein at various pH values at 29.5 C. ○ Viscosity with no added salts; ● viscosity with 0.033 M NaCl present; △ viscosity with 0.016 M $CaCl_2$ in addition to NaCl; ▲ viscosity with 0.0063 M Na_2HPO_4 in addition to $CaCl_2$ and NaCl.

the solutions are white and firm gels are obtained. Maximum micelle formation, mediated by $CaCl_2$, must be obtained for the gel-forming properties of phosphate to be evident.

The contribution of the casein is direct. When the concentration is decreased to 0.26%, gels are obtained but they are much weaker and break when the tubes are inverted.

The phosphate effected gel formation from 0.00042 to 0.0102 M, with molar ratios of phosphate to $CaCl_2$ of 1:24 to 1:1. At the lowest concentration of phosphate the gels, although formed, were not as strong as at the highest concentrations. A substituted phosphate, glycerophosphate, was not effective in gel formation. Although esterification of orthophosphate leads to increased acid strength of the primary and secondary acid groups (5), the change in acid strength is probably not the responsible factor. It is more likely that the substituted glycerol group sterically hinders the phosphate-calcium bonding. Polyphosphate (7) prevented micelle formation, presumably by binding Ca^{++}; therefore, the influence on gel formation could not be determined. Imidazole (0.02 M) weakened gel strength but again a reduction in micelle formation was apparent. This may have been from competition with the calcium ions, already noted (8), since the solutions were less opalescent than with $CaCl_2$ alone.

Because of the probable ionic nature of the phosphate effect, the influence of NaCl on this was determined. Unfortunately, the NaCl effected micelle formation, judged by reduction in turbidity, before any effect on the phosphate gel formation was noted. Accordingly, it could only be concluded that the phosphate-calcium bond was no weaker than the calcium-casein bond.

The lack of influence of calcium and phosphate ions on the whole casein gel by HCl vapors is not surprising, since they are not bound to the caseins at an acidic pH (13).

The weak gels formed when rennin acts on κ-casein are developed independently of the kind of salt. This suggests that formation of these gels is an inherent property of the κ-casein that becomes manifest when transformed to para-κ-casein which cross-bonds to form the gels. Presumably, salts strengthen the gels by reducing electrostatic repulsion.

The lack of a specific phosphate effect with κ-casein compared with whole casein, taken with the inability to demonstrate a phosphate cross-bonding by viscosity measurements before rennin acts, suggests that the action of rennin on the casein micelle produces or exposes a phosphate reactive component. The action of rennin on κ-casein, if it is on the surface of the micelle, could lead, by the physical removal of the

glycomacropeptide area or by formation of a network of strands of para-κ-casein, to the exposure of a surface, presumably α_s- or β-casein, permitting calcium-phosphate cross-bonding and a firm gel structure. An alternative explanation (I am indebted to Dr. Richard M. Parry, Jr., for this suggestion) for the specific phosphate effect arises from the cationic nature of the para-κ-casein. Separation of the acidic glycomacropeptide from the micelle, already essentially isoelectric becouse of bound calcium ions, could increase the positive charge such that a cross bond of phosphate-calcium-phosphate could arise. This could also occur through hydrophobic association of a para-κ-casein aggregate to the micelle and this weak positively charged area would give rise to phosphate-calcium salt bridges.

The reduction of viscosity of casein solutions by NaCl is well known (2, 4, 12) and can be attributed to the decrease of electrostatic charge repulsion within the molecule. The influence of the calcium ion, however, is a specific effect leading to micelle formation and concomitant reduction in viscosity. The parallel changes in both micelle formation and viscosity indicate that the micelles are more compact structures than the unaggregated molecules. That addition of phosphate does not increase viscosity supports the aforementioned theory that the surface of the micelle does not favor calcium-phosphate cross bonds but that the action of rennin leads to the appearance of a reactive surface. The slight reduction in viscosity with phosphate suggests that the calcium-produced casein micelle becomes more compact on the addition of phosphate.

References

(1) Hammarsten, O. 1896. Über de Verhalten des Paracaseins zu dem Labenzym. Z. physiol. Chem., 22: 103.

(2) Hankinson, C. L., and D. R. Briggs. 1941. Electrokinetics. XXV. The electroviscous effect. II. In systems of calcium and sodium caseinates. J. Phys. Chem., 45: 943.

(3) Scott Blair, G. W., and J. C. Oosthuizen. 1961. A viscometric study of the breakdown of casein in milk by rennin and rennet. J. Dairy Res., 28: 165.

(4) Scott Blair, G. W., and J. C. Oosthuizen. 1962. Viscometric measurements of the activities of commercial rennets using sodium caseinate as substrate. J. Dairy Res., 29: 37.

(5) Zittle, C. A. 1948. Manometric determination of phosphomonoesterase (alkaline phosphatase). J. Franklin Instit., 245: 265.

(6) Zittle, C. A. 1966. Precipitation of casein from acidic solutions by divalent anions. J. Dairy Sci., 49: 361.

(7) Zittle, C. A. 1967. Precipitation of caprine and bovine caseins from acidic solutions by sodium polyphosphate: Influence of pH and urea. Utilization for separation of α_s- and κ-caseins. J. Dairy Sci., 50: 1352.

(8) Zittle, C. A. 1969. Influence of heat on κ-casein. J. Dairy Sci., 52:12.

(9) Zittle, C. A., and J. Cerbulis. 1958. Clotting of casein with pepsin: Amount and nature of the soluble products. J. Dairy Sci., 41: 241.

(10) Zittle, C. A., J. Cerbulis, L. Pepper, and E. S. Della Monica. 1959. Preparation of calcium-sensitive α-casein. J. Dairy Sci., 42: 1897.

(11) Zittle, C. A., and J. H. Custer. 1963. Purification and some of the properties of α_s-casein and κ-casein. J. Dairy Sci., 46: 1183.

(12) Zittle, C. A., E. S. Della Monica, and J. H. Custer. 1956. Precipitation of calcium caseinate by heat and subsequent reversal. J. Dairy Sci., 39: 1651.

(13) Zittle, C. A., E. S. Della Monica, R. K. Rudd, and J. H. Custer. 1958. Binding of calcium to casein: Influence of pH and calcium and phosphate concentrations. Arch. Biochem. Biophys., 76: 342.

(14) Zittle, C. A., M. P. Thompson, J. H. Custer, and J. Cerbulis. 1962. κ-Casein-β-lactoglobulin interaction in solution when heated. J. Dairy Sci., 45: 807.